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Clustering of Trichothecene-Producing *Fusarium* Strains Determined from 28S Ribosomal DNA Sequences

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The genus *Fusarium* includes several species that produce trichothecenes. We analyzed DNA sequences from a variable region at the 5′ end of the large nuclear ribosomal DNA (rDNA) (28S) to determine the genetic relatedness of trichothecene-producing *Fusarium* species. All trichothecene-producing strains clustered together, and two monophyletic groups were resolved. The first clade included strains of *F. acuminatum*, *F. sambucinum*, *F. tumidum*, *F. compactum*, *F. camptoceras* (red pigment), *F. sporotrichioides*, and *F. venenatum*, which produced type A trichothecenes (T-2 toxin, HT-2 toxin, neosolaniol, and diacetoxyscirpenol). The second clade consisted of strains of *F. crookwellense*, *F. culmorum*, and *F. graminearum*, which produced type B trichothecenes (fusarenone-X, nivalenol, and deoxynivalenol). The phylogenetic placement of the species based on rDNA correlated better with toxic secondary metabolite data rather than with the current classification system based on morphology.

Trichothecene toxins are produced by several species of *Fusarium*. Trichothecenes are a closely related group of sesquiterpenes that are potent eukaryotic protein synthesis inhibitors and are often associated with a variety of animal and human mycotoxicoses (18). Chemically, *Fusarium* trichothecenes have been classified based on the substituent functional groups, and they can be characterized by the presence or absence of a keto group at the C-8 position. Consistent differences in toxigenicity exist between type A and B trichothecenes (28).

In spite of their similar biosynthetic abilities, trichothecene-producing *Fusarium* species have been placed in various sections of the genus. The species reported as major producers of type A trichothecenes (e.g., T-2 toxin [T-2], HT-2 toxin [HT-2], neosolaniol [NEOS], and diacetoxyscirpenol [DAS]) are *F. sambucinum* Fuckel, *F. venenatum* Nirenb. (*Discolor* Woll. section) (1, 18), *F. acuminatum* Ell & Ev., *F. compactum* (Woll.) Gordon (*Gibbosum* Woll. section) (12, 29), and *F. sporotrichioides* Sherb. (*Sporotrichiella* Woll. section) (14, 18). The majority of strains producing type B trichothecenes (e.g., nivalenol [NIV], fusarenone-X [FUS], and deoxynivalenol [DON]) are *F. crookwellense* Burgess, Nelson & Toussoun (6, 8), *F. culmorum* (W. G. Smith) Sacc., and *F. graminearum* Sch. (13, 18), all part of the *Discolor* section. Many *Fusarium* species are not well described, however, either because they have not been intensively studied or because they include only a few atypical strains that can produce trichothecenes. For example, atypical strains of a relatively rare population of *F. camptoceras* Wollenw. & Reink. (*Arthrosporiella* Woll. section) with red pigmentation and strains of *F. tumidum* Sherb. (*Discolor* Woll. section), a "not well documented" species (21), were recently reported to produce trichothecenes (2, 15).

Our objective in this study was to examine the genetic relatedness of trichothecene-producing *Fusarium* species, through sequence analysis of a portion of the ribosomal DNA (rDNA) coding region. A short, highly variable region of the large-subunit rDNA has been used for phylogenetic studies in lower eukaryotes (4), including various fungal genera (9, 19, 23, 26). In a preliminary study, we found a possible correlation between phylogenetic affinities obtained by sequencing the 5′ end of the region coding for the large-subunit rRNA and the production of trichothecenes (16). In this report, we identify a phylogenetic affinity within *Fusarium* trichothecene-producing species, based on DNA sequences at the 5′ end of the larger nuclear rDNA.

MATERIALS AND METHODS

Fungal strains. The strains used in this study are described in Table 1. Before further study, they were stored on slants of special nutrient agar (22) at 4°C.

Chemical analysis. We tested all of the strains for their ability to produce trichothecenes. The strains were grown on 50 g of maize kernels var. Plata, which had been brought overnight to approximately 45% moisture in 250-ml Erlenmeyer flasks and then autoclaved for 30 min at 120°C. The substrate was inoculated with 2 ml of approximately 10^7 conidia/ml. The cultures were shaken once daily for 3 days to distribute the inoculum and were incubated at 25°C in the dark for 4 weeks. The harvested culture material was dried in a forced-air oven at 50°C for 48 h, finely ground in a Waring blender, and stored at 4°C until use. As a control, uninoculated cornmeal was used.

For extractions, culture material (20 g) was extracted with 100 ml of methanol–1% aqueous NaCl (55:45, vol/vol) in a blender for 3 min and filtered through paper (Whatman no. 1) under vacuum at room temperature. The filtrate (50 ml) was defatted with *n*-hexane (50 ml per extraction for three extractions) and then extracted exhaustively with dichloromethane (30 ml per extraction for three extractions). The organic extracts were collected and evaporated to dryness under reduced pressure at room temperature (24 to 28°C), and the residue was dissolved in 1 ml of methanol. The qualitative analyses of trichothecenes of type A (T-2, HT-2, NEOS, and DAS) and type B (NIV, FUS, and DON) were performed by thin-layer chromatography and high-performance thin-layer chromatography (5). The detection limit for each trichothecene was approximately 1 μg/g of dried corn culture. Toxin reference standards were purchased from Sigma Chemical Co. (St. Louis, Mo.).

rDNA sequences. DNA for PCR was extracted by a modified version of the sodium dodecyl sulfate protocol of Raeder and Broda (25). Fresh mycelium (≈50 mg) was scraped from a petri dish culture, resuspended in 600 μl of extraction buffer (200 mM Tris [pH 8.4], 200 mM EDTA [pH 8.0], 0.5% sodium dodecyl sulfate) in an Eppendorf tube, and incubated for 1 h at 37°C. The slurry was homogenized twice with 600 μl of phenol and centrifuged for 10 min in an Eppendorf centrifuge (at 1,300 × *g*). The upper, aqueous phase was extracted with 600 μl of chloroform and centrifuged as described above. DNA was precipitated by addition of 2.5 volumes of 95% ethanol and 1/10 volume of 3 M sodium acetate. The samples were centrifuged for 20 min at 1,300 × *g*, and the DNA pellet was recovered and dissolved in 50 μl of sterile water. The PCR was set up with 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim Biochemicals) in 100-μl reaction mixtures containing 50 pmol of each outside primer, F65

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TABLE 1. *Fusarium* strains used in this study

Fusarium species	Section	Strain no.	Host plant and/or origin	Trichothecene(s) produced	EMBL accession no.	Reference
Trichothecene producers						
Type A						
F. acuminatum	*Gibbosum*	NRRL-6227[a]	*Festuca* sp., United States	T-2, HT-2, NEOS	X80239	18
F. acuminatum	*Gibbosum*	KF-359[b]	Poland	T-2		12
F. acuminatum	*Gibbosum*	MRC-3826[c]	*Avena* sp., South Africa	T-2		12
F. camptoceras (R)	*Arthrosporiella*	ITEM-1138[d]	*Musa* sp., Panama	T-2, HT-2	X80803	15
F. compactum	*Gibbosum*	R-6784[e]	River sediments, Japan	T-2, NEOS	X80804	18
F. sambucinum	*Discolor*	64995[f]	*Brassica* sp., Netherlands	DAS, NEOS	X80805	1
F. sporotrichioides	*Sporotrichiella*	NRRL-3299[a]	*Zea mays*, France	T-2, HT-2, DAS	X80806	18
F. sporotrichioides	*Sporotrichiella*	ITEM-390[b]	*Zea mays*, Italy	T-2		14
F. tumidum	*Discolor*	NRRL-13394[a]	*Lupinus* sp., New Zealand	NEOS	X80807	2
F. venenatum	*Discolor*	64935[f]	*Solanum tuberosum*, Poland	DAS	L28694[g]	1
Type B						
F. crookwellense	*Discolor*	KF-748[b]	*Solanum tuberosum*, Poland	FUS, NIV	X80808	8
F. crookwellense	*Discolor*	ITEM-619[d]	*Triticum* sp., Yugoslavia	FUS		6
F. culmorum	*Discolor*	NRRL-3288[a]	Unknown	DON	X80809	18
F. culmorum	*Discolor*	ITEM-328[d]	*Triticum durum*, Italy	DON		13
F. culmorum	*Discolor*	ITEM-628[d]	*Triticum* sp., Yugoslavia	DON		13
F. graminearum group I	*Discolor*	R-6710[e]	Unknown	DON	X80810	
Trichothecene nonproducers						
F. camptoceras (B)	*Arthrosporiella*	ITEM-1128[d]	*Musa* sp., Ecuador	ND[h]	X80811	15
F. equiseti	*Gibbosum*	NRRL-13405[a]	Unknown	ND	X80812	
F. semitectum	*Arthrospor*	NRRL-13327[a]	Unknown	ND	X80813	
F. tricinctum	*Sporotrichiella*	T-429[e]	*Hordeum* sp., Germany	ND	X80814	18
F. decemcellulare	*Spicarioides*	NRRL-13411[a]	Unknown	ND	X80815	
F. solani	*Martiella*	NRRL-13417[a]	Unknown	ND	X80816	

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[g] Sequence obtained from GenBank data.
[h] ND, not detected.

(5′-GCATATCAATAAGCGGAGGAAAAG) and R635 (5′-GGTCCGTGTTTCAAGACG), 1.25 mM deoxynucleoside triphosphates (Perkin-Elmer Cetus), and 1 μl (approximately 5 ng) of fungal template DNA. The reactions were cycled (Perkin-Elmer Thermocycler) 30 times (1 min at 94°C, 1 min at 52°C, and 2 min at 72°C). Control tubes without the DNA template were included in each experiment (negative control). After amplification, a 10-μl aliquot from each sample was subjected to electrophoresis.

From the samples containing sufficient DNA, excess primer and deoxynucleoside triphosphates were removed with a Qiaex gel extraction kit (Qiagen, Chatsworth, Calif.) as specified by the manufacturer. Single-stranded DNA templates were sequenced completely on both strands with the chain-terminating dideoxynucleotide Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, Ohio) and [^{32}P]dATP. Two sequencing reactions were run on each template, one in which the template was primed with 10 pmol of an external limiting primer (R635) and the second in which the template was primed with 10 pmol of an internal primer (5′-GATGAAAAGCACTTTGAAAAGAG). Sequencing reactions were run on 6% polyacrylamide Wedge gels for approximately 3 h, fixed, vacuum dried, and exposed overnight to Kodak Ektamat-G film.

Construction of the phylogenetic tree. The phylogenetic tree was constructed by using a stationary Markov model (27). This method permits the calculation of time-of-divergence ratios (t/t') for any pair of homologous sequences by analyzing any class of site, which is assumed to be subjected to the same evolutionary dynamics.

Nucleotide sequence accession numbers. The sequences at the 5′ end of the 28S rDNA have been deposited with the EMBL database. The accession numbers are given in Table 1.

RESULTS

A 239-base region at the 5′ end of the 28S rDNA was sequenced on both strands for all the strains listed in Table 1. These sequences have been deposited with the EMBL database or may be obtained collectively in an aligned format from the first author. No intraspecific sequence variation was found in *F. acuminatum*, *F. sporotrichioides*, *F. crookwellense*, and *F. culmorum*. Trichothecene-producing strains clustered together, and two principal monophyletic groups were resolved (Fig. 1). The first clade included strains of *F. acuminatum*, *F. sambucinum*, *F. tumidum*, *F. compactum*, *F. camptoceras* (red), *F. sporotrichioides*, and *F. venenatum*, which produced type A trichothecenes. The second clade consisted of strains of *F. crookwellense*, *F. culmorum*, and *F. graminearum*, which produced type B trichothecenes.

The sequences of the six type A trichothecene-producing species differed from each other by no more than 2.5% (1/239 to 6/239 [number of differences/total number of aligned sites]). *F. tumidum* NRRL-13394 and *F. sambucinum* 64995 had the same sequence in this region. Interspecific nucleotide differences among trichothecene type B producers also were very low. *Fusarium crookwellense* and *F. culmorum* had the same sequence, and these species differed from *F. graminearum* group I (R-6710) by 2 bases (0.8%). Not surprisingly, the trichothecene-nonproducing strains are more diverse than is either group of trichothecene-producing strains (2.0 to 10.4%). The two *F. camptoceras* strains (ITEM-1138 and ITEM-1128) had quite different (9/239 bp, 3.7%) sequences, a result that is consistent with their abilities to produce pigments and mycotoxins. *F. camptoceras* ITEM-1138 (T-2 and NEOS producer)

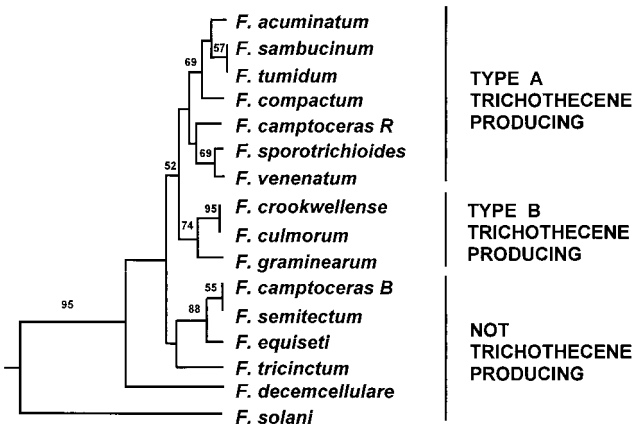

FIG. 1. Phylogenetic relationship of the toxigenic *Fusarium* species, inferred from the nucleotide sequence of the 5′ end of large-subunit rDNA. The phylogenetic tree was constructed by using the stationary Markov model. The number at each fork indicates the percentage of times that the group consisting of the species to the right of that fork occurred among the trees. The number at the forks is omitted when lower than 50.

was genetically related to other type A trichothecene-producing strains (sequence divergence of 0.8 and 2.5% from *F. venenatum* 64935 and *F. compactum* R-6784, respectively). On the other hand, *F. camptoceras* ITEM-1128 (trichothecene nonproducer) was genetically related to trichothecene nonproducing strains, particularly *F. semitectum* NRRL-13327.

DISCUSSION

rRNA coding sequences have been used to establish phylogenetic relationships. The advantages of using these sequences for phylogenetic studies are that this molecule is universally present in all living cells and that its sequence is not correlated with a particular morphology. It is known, however, that not all portions of the molecule are equally suitable for detecting molecular changes, as different portions of the molecule are subject to different selection pressures. The structural features shared by 28S-like molecules in all species provides a versatile and sensitive phylogenetic indicator, since highly conserved sequences are interspersed with much more rapidly evolving domains (7, 10).

In this study, we found that the 5′ region we sequenced is relatively well conserved in all 15 *Fusarium* species examined. The nucleotide changes that have occurred can be used to divide the trichothecene-producing *Fusarium* strains into two distinct clusters.

In a dendrogram (Fig. 1) based on our data, *F. solani* and *F. decemcellulare* are unequivocally separated (bootstrap interval, 95) from the other species analyzed, in agreement with their distinct teleomorph states (21). The remaining strains fall into three distinct clusters that correlate with their ability to synthesize trichothecenes. Although *F. acuminatum*, *F. camptoceras* (R), *F. compactum*, *F. sporotrichioides*, *F. sambucinum*, *F. tumidum*, and *F. venenatum* are morphologically distinct species that belong to four different *Fusarium* sections, on the basis of sequence analysis they are more closely related to each other than they are to other members of the same section that cannot synthesize type A trichothecenes. The phylogenetic relationship between *F. acuminatum* and *F. sporotrichioides* was also supported by comparing their electrophoretic karyotypes (20). In addition, Altomare et al. (3) investigated the taxonomic relationships among *F. acuminatum* subspp. *acuminatum* and *armeniacum*, *F. sporotrichioides*, and *F. tricinctum* by isozyme analysis and found that the trichothecene-producing strains of *F. acuminatum* were more closely related to *F. sporotrichioides* than to the trichothecene-nonproducing strains of *F. acuminatum*. The correlation between trichothecene production and rDNA sequence and the lack of correlation with traditional morphological characters suggest that a major revision of the systematics of this genus is needed if the systematics are to be consistent with the phylogeny. In addition, the DNA sequence data from this region could be used to design a PCR primer pair which specifically amplifies DNA from trichothecene-producing *Fusarium* strains.

To our knowledge, this is the first report of a correlation between the phylogenetic placement of a large number of toxigenic *Fusarium* species belonging to different sections and their trichothecene production. Correlations between genetic data and secondary metabolite profiles are known to occur in other toxigenic genera. For example, studies examining the taxonomic position of some toxigenic *Penicillium* species inferred from the phylogenetic analysis of rDNA sequences, including the region used in this study, found that rDNA sequences and mycotoxin profiles were correlated (11, 17, 24). In conclusion, we found that the variable region of the 5′ end of the 28S large nuclear rDNA was useful for taxonomic purposes, correlated with mycotoxin production potential, and could provide the basis for identification of uncertain and atypical toxigenic *Fusarium* strains.

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